Review

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# Integration of guidance cues: parallel signaling and crosstalk

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**Growing axons are exposed to various guidance cues en route to their targets. Although many guidance molecules have been identified and their effects on axon behavior extensively studied, how axons react to combinations of signals remains largely unexplored. We review recent studies investigating the combined actions of guidance cues present at the same choice points. Two main scenarios are emerging from these studies: parallel signaling and crosstalk between guidance systems. In the first case, cues act in an additive manner, whereas in the second case the outcome is non-additive and differs from the sum of individual effects, suggesting more complex signal integration in the growth cone. Some of the molecular mechanisms underlying these interactions are beginning to be unraveled.**

## Introduction

A functional nervous system is assembled during development by different neuronal populations connecting to each other in a highly selective manner. A stereotyped wiring pattern is first established according to a genetic program that specifies the complement of axon guidance receptors expressed by each neuronal population. This stereotyped pattern is subsequently refined through neuronal activity. During the initial, activity-independent wiring phase, axons are steered towards the correct synaptic partner cells by attractive and repulsive molecular cues along their path. Several families of axon guidance cues and receptors have been described in the past two decades [1]. Their effects on different populations of axons have been studied in a number of *in vitro* assays and confirmed in different model organisms *in vivo*. Most of the studies so far have addressed the functions of a certain guidance cue presented in isolation. However, when axons navigate their way *in vivo*, at every choice point they are probably confronted with several different signals acting in a permissive or instructive way. How are multiple signals integrated by the growth cones to bring about the correct path-finding decisions?

Here we review recent studies that have begun to address this question by examining combinations of cues that control guidance choices at the same decision points. Although our understanding of signal integration by the growth cone is still very incomplete, there seem to be two main scenarios. First, different cues might exert their actions independently (or in parallel). They might act in a cooperative or opposing manner, depending on the nature of the respective molecules and their spatial distribution, but the net result amounts to the sum of the effects of individual molecules. Second, guidance systems might interact in a non-additive manner, with the net outcome being different from the sum of isolated effects. Crosstalk between signaling pathways can happen at the level of the ligands, their receptors on the membrane, or their intracellular signaling effectors. In some cases one of the cues appears to be dominant and suppresses the activity of the other one. Conversely, cues might interact in a synergistic way, promoting the same growth cone behavior. Finally, there are examples of permissive interactions, whereby exposure to one (permissive) factor enables the axons to sense the other (instructive) signal that provides directional information. Surprisingly, even the same combination of cues can lead to different outcomes depending on the cellular context. Therefore, it is becoming clear that interactions of multiple signals are usually more complex than a simple balance of attractive and repulsive influences at each decision point.

In this review we focus on studies dealing with the most widely used and relatively well understood model systems in vertebrates, with an emphasis on the most recent literature. A more comprehensive list of known interactions is presented in Table 1.

## Additive effects of guidance cues

One of the systems in which several cues were shown to act in an additive manner is the motor axon projection to the vertebrate hindlimb. Limbs receive motor innervation from the neurons of the lateral motor column (LMC), located at the brachial and lumbar levels of the spinal cord. Within the LMC, the lateral subpopulation of cells ($LMC_L$) projects to the dorsal compartment of the limb, whereas the medial subdivision ($LMC_M$) projects to the ventral compartment (Figure 1a) [2]. EphrinAs and glial-cell-derived neurotrophic factor (GDNF) both control the dorsal or ventral pathway choice of certain limb-innervating motor axons. Their respective receptors, EphA4 and Ret, are enriched in dorsally fated $LMC_L$ axons. In the limb mesenchyme, ephrinAs are expressed on the ventral side and elicit repulsive EphA signaling in axons, pushing them into the dorsal pathway [3,4]. The Ret ligand GDNF is present dorsal to the choice point and guides $LMC_L$ axons by an attractive and growth-promoting mechanism

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**Table 1. Guidance cue interactions**

| Mode of interaction | Guidance cues | Guidance decision | Effects of the cues | Refs |
|---|---|---|---|---|
| Parallel signaling | EphrinAs and EphAs/GDNF | Guidance of $LMC_L$ axons to the dorsal limb | EphrinAs repel axons from the ventral limb, GDNF and EphAs attract axons towards the dorsal limb | [3–10] |
| | Sema3F, ephrinBs, and EphBs | Guidance of $LMC_M$ axons to the ventral limb | Sema3F and ephrinBs repel axons from the dorsal limb[a], EphBs probably attract axons towards the ventral limb[b] | [7,45,63] |
| | Netrin-1, Shh, and VEGF | Guidance of pre-crossing commissural axons towards the ventral midline in the spinal cord | Netrin-1, Shh, and VEGF attract axons towards the floor plate | [13–15,19] |
| | BMPs and Draxin | Guidance of pre-crossing commissural axons towards the ventral midline in the spinal cord | BMPs and Draxin repel axons from the dorsal spinal cord | [64–66] |
| | Slits, Sema3s, and SCF | Expulsion of post-crossing commissural axons from the spinal cord midline | Slits and Sema3s repel the axons from the midline, SCF stimulates growth | [25–27] |
| | EphrinB2 and Shh | Ipsilateral RGC projection at the optic chiasm | EphrinB2 and Shh repel ipsilateral axons from the midline | [29,30,32] |
| | VEGF and Sema6D/ plexin-A1/NrCAM | Contralateral RGC projection at the optic chiasm | VEGF and the tripartite Sema6D/plexin-A1/NrCAM complex promote crossing of contralateral axons | [33,35] |
| | Wnt3 and ephrinB1 | Medial–lateral mapping of retinotectal projections in the final target | Attractive ephrinBs promote medial branch formation, repulsive Wnt3 favors lateral branch formation | [67,68] |
| | Draxin and Tsukushi | Guidance of the corpus callosum and anterior commissure axons across the midline | Draxin and Tsukushi guide forebrain commissural axons by surround repulsion | [69] |
| Hierarchical interactions | Netrin-1 and Slit | Expulsion of post-crossing commissural axons from the spinal cord midline | Silencing of netrin-1/DCC attraction by Slit/Robo | [37,38] |
| | Netrin-1 and Slit | Exit of motor axons from the spinal cord | Silencing of netrin-1/DCC attraction by Slit/Robo | [38] |
| | Netrin-1 and Slit | Guidance of longitudinal tracts along the midline in zebrafish | Probably silencing of netrin-1/DCC attraction by Slit/Robo | [70] |
| | Netrin-1 and Slit | The choice of anterior dorsal telencephalon axons in zebrafish to extend into the anterior commissure or the ipsilateral supraoptic tract | Silencing of netrin-1 attraction by Slit/Robo | [71] |
| | Netrin-1 and Slit | Guidance of callosal axons towards the midline | Silencing of Slit/Robo repulsion by netrin-1/ DCC | [41] |
| | Sema3A and Sema3C | Guidance of corticofugal axons in the intermediate zone of the cortex | Sema3A repels axons from the subventricular and ventricular zones, Sema3C attracts them into the intermediate zone | [43] |
| | Sema3A/3C and Sema3F | Surround repulsion of limb motor axon tracts | Preferential responsiveness to Sema3A/3C or Sema3F expressed on different sides of the nerve depends on regulation of neuropilin receptors by co-expressed Sema3C | [46] |
| Synergistic interactions | GDNF and EphAs | Guidance of $LMC_L$ axons to the dorsal limb | Synergistic growth-promoting effects of GDNF and EphAs through the common signaling receptor Ret | [9] |
| | BMP7 and GDF7 | Repulsion of commissural axons from dorsal spinal cord | BMP7 and GDF7 form a heterodimer with enhanced repulsive activity | [65] |
| Permissive interactions | Sema3B and NrCAM/GDNF/Shh | Expulsion of post-crossing commissural axons from the spinal cord midline | NrCAM, GDNF, and Shh switch on axon responsiveness to Sema3s | [47–49] |
| | Sema6D and plexin-A1/NrCAM | Guidance of crossed RGC projection at the optic chiasm | Plexin-A1 and NrCAM convert the inhibitory action of Sema6D into a growth-promoting effect | [35] |
| | Netrin-1 and Slit1 | Rostral thalamocortical projection | Slit1 confers responsiveness of rostral thalamocortical axons to netrin-1 attraction | [50] |
| | Sema3A and NF-protocadherin | Guidance of *Xenopus* RGC axons in the mid-optic tract | Sema3A prevents RGC axons from entering the telencephalon; in addition, Sema3A/neuro pilin-1 signaling induces local protein synthesis of NF-protocadherin, which guides RGC axons by adhesive homophilic interactions with NF-protocadherin in the substrate tissue | [72] |

[a]It is not clear whether Sema3F and ephrinBs are involved in regulating the same pathway choice, because the role of Sema3F was demonstrated in the forelimb and the role of ephrinBs in the hindlimb.

[b]Attractive effects of EphBs on $LMC_M$ axons have only been demonstrated *in vitro*. Abbreviations: GDNF, glial-cell-derived neurotrophic factor; $LMC_L$, lateral motor column, lateral subdivision; $LMC_M$, lateral motor column, medial subdivision; Shh, sonic hedgehog; VEGF, vascular endothelial growth factor; BMP, bone morphogenic protein; SCF, stem cell factor; RGC, retinal ganglion cell; NrCAM, Ng-CAM-related cell adhesion molecule; DCC, deleted in colorectal cancer; GDF, growth differentiation factor.

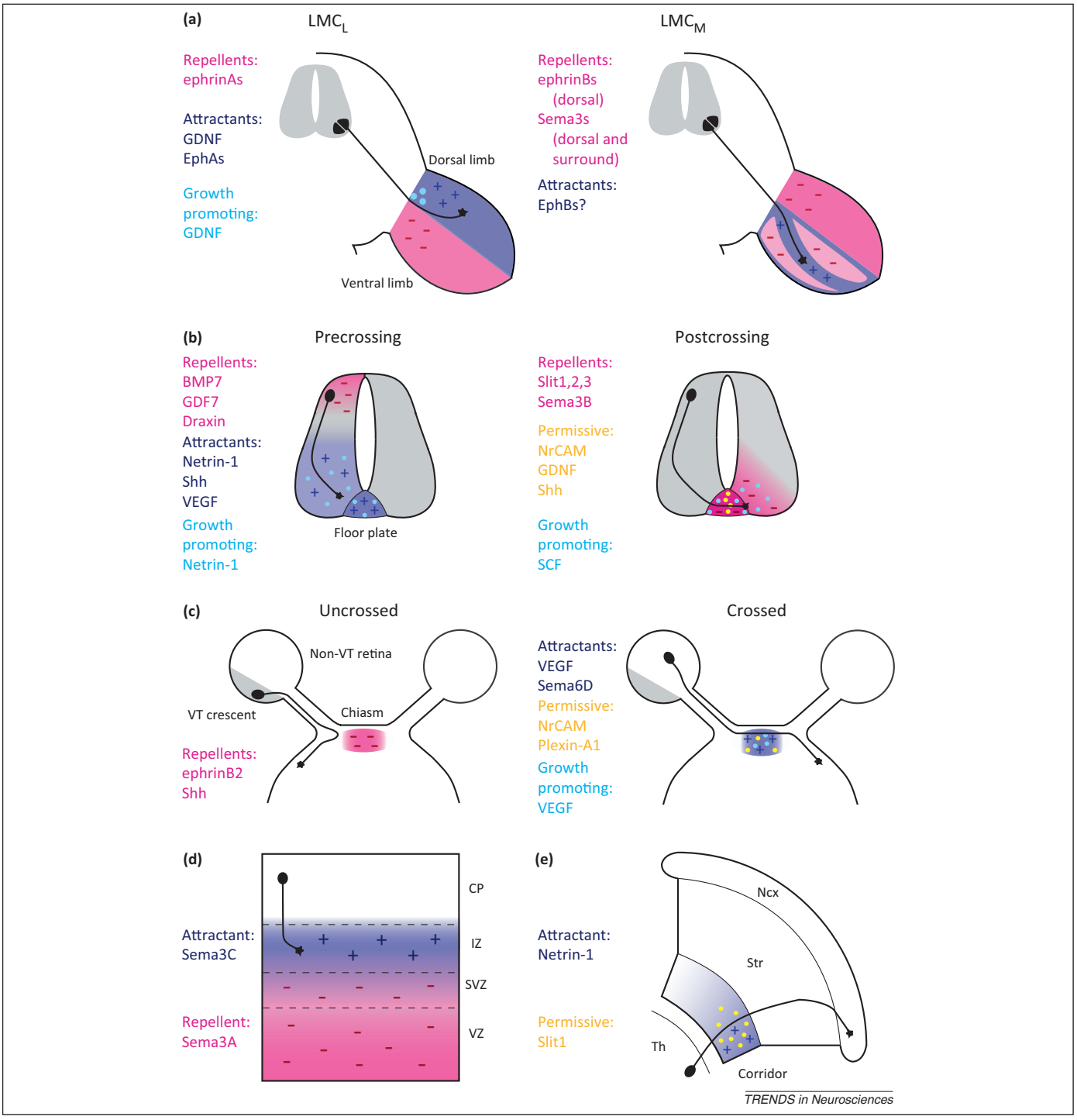


**Figure 1**. Model systems used to study guidance cue interactions. **(a)** Motor axon guidance in the vertebrate limb. Left: the LMC$_L$ projection is guided towards the dorsal limb by a combination of ventral repellents (pink) and dorsal attractants (blue) and a growth-promoting factor (light blue dots). Right: dorsal repulsive cues (pink) guide LMC$_M$ axons toward the ventral limb, where the axons are channeled into their pathway by surround repellants (light pink). *In vitro* studies suggest that EphBs expressed in the ventral limb might also act as attractants (blue) [7]. LMC$_L$, lateral cells of the lateral motor column; LMC$_M$, medial cells of the lateral motor column. **(b)** Midline crossing by commissural axons in the vertebrate spinal cord. Left, commissural axons are first guided towards the floor plate by dorsally expressed repellents (pink gradient) and ventrally expressed attractants (blue gradient) and growth-promoting factors (light blue dots). Right, at the midline, axons are exposed to permissive factors (yellow dots), which sensitize them to midline repellents. Repulsive cues (pink) then expel axons from the floor plate, whereas stem cell factor (SCF; light blue dots) stimulates axon extension. **(c)** Guidance of uncrossed and crossed retinal projections at the optic chiasm. Left, axons of retinal ganglion cells from the ventrotemporal (VT) crescent are guided by midline chemorepellents (pink), which prevent them from crossing the chiasm. This population of axons forms the ipsilateral (uncrossed) projection. Right, axons arising from non-VT parts of the retina form the contralateral (crossed) projection, which is directed across the midline by attractive (blue) and growth-promoting (light blue dots) cues. In addition, permissive molecules (yellow dots) are required for Sema6D to act as an attractant. **(d)** Guidance of corticofugal axons. Corticofugal axons are attracted to Sema3C (blue) expressed in the intermediate zone (IZ) of the developing cortex and repelled from Sema3A (pink) expressed in the ventricular (VZ) and subventricular (SVZ) zones [43]. CP, cortical plate. **(e)** Guidance of the rostral population of thalamocortical axons in the corridor. Slit1 acts as a permissive factor (yellow dots), which enables the axons' attraction to netrin-1 (blue gradient) [50]. As a consequence rostral axons navigate through the rostral striatum and neocortex. Th, thalamus; Str, striatum; Ncx, neocortex.

(Figure 1a) [5,6]. Both Ret and EphA4 knockouts show misprojections of a subpopulation of dorsally fated axons into the ventral hindlimb, a phenotype that is enhanced in Ret/EphA4 double mutants [3,5]. Further experiments showed that the two receptors do not interact with each other and do not regulate each other's expression [5,6]. Moreover, attractive GDNF and repulsive ephrinAs have additive effects on $LMC_L$ axon turning when presented in opposing gradients according to their *in vivo* expression in the dorsal and ventral limb mesenchyme, respectively [6]. All these observations suggest that the two guidance systems operate independently (Figure 2).

In addition, reverse signaling by membrane-bound ephrinAs also acts in parallel with forward EphA4 signaling at this decision point. EphrinAs are expressed along with EphAs in limb motor axons, with the amount of ephrinAs determining the prevalence of ligand–receptor *cis*-interactions on the axonal membrane. In the $LMC_L$ population, EphAs and ephrinAs are mostly free from *cis*-interactions and segregate into different membrane domains [7]. This enables them to independently transduce forward and reverse signals on binding of their interaction partners in *trans* [7,8]. In the hindlimb, EphAs are restricted to the dorsal compartment and attract $LMC_L$ axons by activating axonal ephrins (Figure 1a) [9,10]. Interestingly, although there is no evidence of crosstalk between GDNF and ephrinAs, GDNF and EphAs expressed in the limb mesenchyme cooperate in activating the Ret receptor (see below) [9].

Several examples of cooperation between guidance systems came from studies of commissural axons in the spinal cord. Commissural neuron cell bodies are situated in the dorsal spinal cord and their axons first grow ventrally towards the floor plate, guided by dorsal repellents and ventral midline attractants (Figure 1b) [11,12]. Three proteins secreted by the spinal cord floor plate, netrin-1, sonic hedgehog (Shh), and vascular endothelial growth factor (VEGF), act as chemoattractants and change the direction of precrossing commissural axon growth *in vitro* [13–15]. Their respective receptors, deleted in colorectal cancer (DCC), Boc, and Flk1, are found on commissural axons [15–18]. Mice deficient in netrin-1, Shh, or VEGF signaling display path-finding errors of precrossing commissural axons [14,15,19]. It should be noted that while all three cues possess chemoattractive activity, netrin-1 has additional growth-promoting properties, allowing axons to invade the ventral spinal cord [14,19,20]. Although genetic data suggest that the three midline chemoattractants likely act in an additive manner, this has not yet been confirmed in quantitative *in vitro* assays after simultaneous presentation of the ligands. It should therefore be kept in mind that further experiments might reveal yet unknown interactions between the cues. For example, their signaling pathways may converge on common downstream effectors. It has been found that Src family kinases are involved in the attractive turning responses to netrin-1 [21–23], as well as Shh [24] and VEGF [15]. Alternatively, one of the cues might regulate the expression, trafficking, activation, or processing of receptors for the other cues.

After arriving in the floor plate, commissural axons acquire sensitivity to the repellent signals present at the midline, such as Slit-1–3 and Sema3B, which expel the axons from the floor plate and push them to the contralateral side (Figure 1b) [25,26]. In mutants lacking Slits or their receptors Robo1 and Robo2, the trajectory of precrossing fibers is not altered, but defasciculation, stalling, recrossing, and misguidance are observed after axons reach the floor plate [26]. Similarly, in the absence of the Sema3 receptor neuropilin-2, axons are guided properly towards the midline, but stall, become disorganized in the floor plate, and show path-finding errors after crossing [25]. In addition to the Slit and Sema3 repellents, stem cell factor (SCF), which is also expressed in the floor plate, is required for midline exit by postcrossing axons. In mice lacking SCF or its receptor Kit, the majority of commissural axons show a clear delay in exiting the midline. Several experiments were performed to test if SCF might act by switching on axon sensitivity to midline repellents. However, SCF failed to alter the responsiveness of commissural axons to floor-plate-derived repellents in *in vitro* assays, and no changes in the expression of axonal receptors such as Robos were observed in SCF or Kit mutants. Therefore, current evidence indicates that these signals act independently: repulsive cues instruct axons to exit the midline and avoid crossing it again, whereas SCF provides a growth impulse that enables axons to leave the floor plate [27].

At the optic chiasm, another model system for investigating guidance mechanisms at intermediate targets, axons of retinal ganglion cells (RGCs) traverse the midline

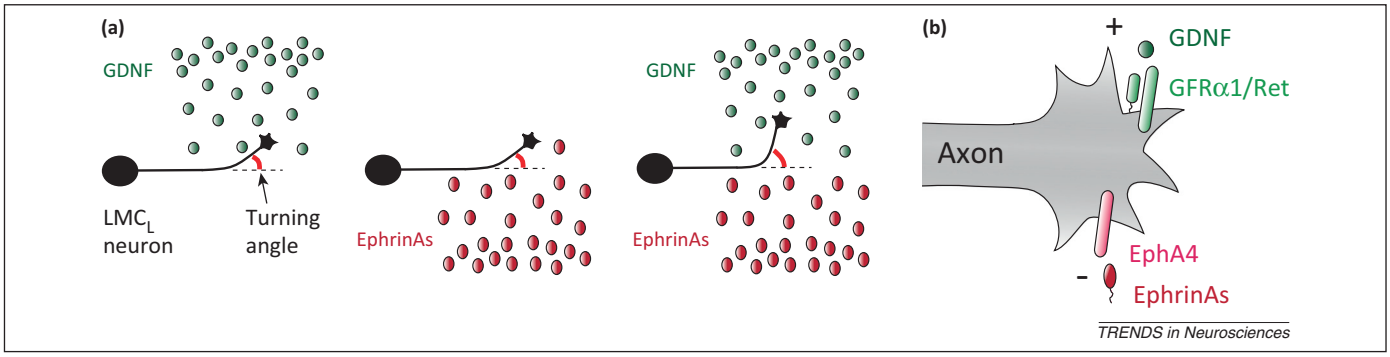


**Figure 2**. Additive effects of glial-cell-derived neurotrophic factor (GDNF) and ephrinAs in motor axons. **(a)** Left: a gradient of GDNF induces attractive turning of $LMC_L$ axons. Middle: repulsive turning is observed in a gradient of soluble ephrinAs. Right: when the two gradients are applied from opposite sides, a more robust turning response is induced. $LMC_L$, lateral cells of the lateral motor column. **(b)** GDNF and ephrinAs exert their effects independently, binding to the GFRα1/Ret and EphA receptors, respectively, on the growth cones [6].

to the contralateral side. In lower vertebrates, all the RGC axons cross and project to the contralateral tectum, whereas in higher vertebrates with binocular vision, a certain population of axons does not cross and continues to grow ipsilaterally. In the mouse, these uncrossed axons originate from RGCs residing in a restricted peripheral area of the retina, the ventrotemporal (VT) crescent (Figure 1c). Multiple molecules expressed at and around the chiasm control the segregation of axons into ipsi- and contralateral projections and direct the contralateral fibers across the midline [28]. Two repulsive midline cues, ephrinB2 and Shh, were implicated in guiding the uncrossed projection (Figure 1c) [29–31]. The ephrinB2 receptor EphB1 and the Shh receptor Boc are both detected in VT cells, and genetic deletion of either EphB1 or Boc results in a markedly reduced uncrossed projection [30,32]. Both receptors are coexpressed in the same RGCs, so it seems unlikely that ephrinB2 and Shh act on separate populations of ipsilateral axons [32]. It remains to be investigated whether any crosstalk exists between the two ligand–receptor pairs.

The signals directing contralateral axons across the chiasm proved more difficult to unravel, but two parallel signaling systems also seem to be involved here (Figure 1c). Similar to the spinal cord midline, one important attractive cue is VEGF; however, it acts through a different receptor, neuropilin-1. It was shown that VEGF164 (the VEGF isoform that binds neuropilin) exerts both chemoattractive and growth-promoting effects on non-VT axons. Accordingly, an increase in the ipsilateral projection was observed in mice with mutant neuropilin-1 and VEGF164 [33]. The second chemoattractive signal is a tripartite complex consisting of Sema6D, plexin-A1 and Ng-CAM-related cell adhesion molecule (NrCAM). These ligands are recognized by a receptor complex comprising plexin-A1 and NrCAM expressed on the crossed population of RGC axons (see below) [34,35]. The uncrossed projection is increased in Sema6D knockouts and NrCAM/plexin-A1 double mutants [35]. It is not known whether the two signaling pathways converge at any level. Neuropilins signal through plexin coreceptors in other contexts [36], so an interesting possibility is that both VEGF164 and Sema6D pathways may use plexins as signaling receptors.

## Non-additive effects of guidance cues

### *Hierarchical interactions*

There is increasing evidence that guidance molecules do not always interact in an additive way. In some cases, one cue might be dominant and suppress responses to the other. Among these hierarchical interactions, the negative regulation of netrin/DCC signaling by Slit/Robo has been investigated in most detail. Early experiments in cultured *Xenopus* spinal neurons showed that attraction to netrin-1

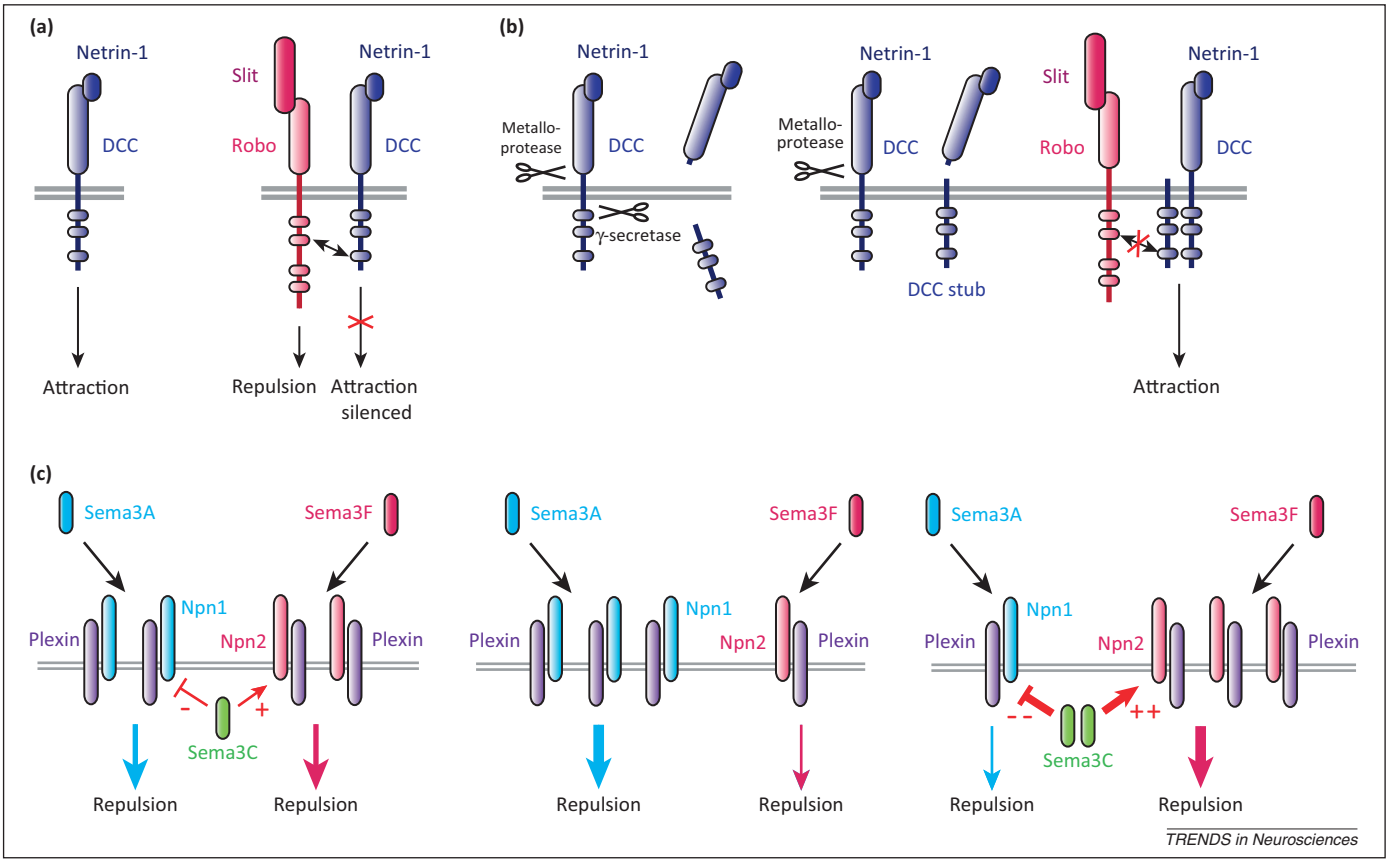


**Figure 3.** Hierarchical interactions between guidance cues. **(a)** Netrin/DCC-mediated attraction is inhibited by Slit/Robo through a direct interaction between Robo and DCC intracellular domains [37]. **(b)** Left: on binding of the netrin ligand, the DCC receptor is removed from the cell surface via sequential cleavage by a metalloprotease and γ-secretase [38–40]. Right: in Columbus mutants, the absence of γ-secretase leads to accumulation of N-terminally truncated DCC receptors (DCC stubs). DCC stubs bind full-length DCC receptors, forming signaling complexes that are immune to Robo silencing. This results in inappropriate attraction to netrin-1 [38]. **(c)** Left: in motor neurons, the balance of Sema3A and Sema3F responses is regulated by Sema3C co-expressed with neuropilin receptors. Sema3C reduces levels of the Sema3A receptor neuropilin-1 and increases levels of the Sema3F receptor neuropilin-2. Sema3C loss-of-function (middle) and gain-of-function (right) manipulations lead to opposing changes in Sema3A and Sema3F sensitivity [46].

was abolished in the presence of Slit [37]. The silencing of netrin attraction was caused by a direct interaction between Robo1 and DCC intracellular domains, which was triggered by Slit binding to Robo (Figure 3a). This interaction is important *in vivo* for the guidance of postcrossing commissural axons, which acquire responsiveness to Slit on traversing the floor plate (Figure 1b). In addition to being a chemorepellent, Slit simultaneously switches off the attraction to netrin-1 expressed in the floor plate, thus facilitating midline exit [37].

Netrin/DCC silencing also seems to be important in spinal motor neurons, which express the DCC receptor, but nevertheless do not grow towards the netrin-1 source in the floor plate and instead leave the spinal cord via ventral roots. This lack of responsiveness to netrin-1 can be explained by the presence of Robo and Slit, which are both expressed by motor neurons and keep DCC in an inactive state [38]. Interesting insights into the regulation of the interplay between Slit/Robo and netrin/DCC signaling came from a mouse mutant called Columbus, generated in a forward genetic screen. In this mutant, many motor neurons fail to exit the spinal cord and instead grow towards the midline because of inappropriate attraction to netrin-1. The mutation was mapped to the gene encoding the γ-secretase presenilin-1 [38]. On binding to netrin, DCC is usually removed from the membrane through consecutive cleavage by a metalloprotease and γ-secretase (Figure 3b) [38–40]. In the absence of presenilin-1 activity, metalloprotease cleavage generates N-terminally truncated DCC receptors (DCC stubs), which are not removed from the membrane. These truncated receptors can signal in a complex with full-length DCC, but are resistant to the Slit/Robo silencing that normally operates in motor neurons. Accumulation of DCC stubs at the membrane therefore results in disinhibition of netrin/DCC signaling, causing midline attraction of motor axons (Figure 3b). The same cleavage mechanism may function in commissural axons, because presenilin mutants also displayed midline stalling and recrossing of commissural fibers, path-finding errors consistent with inappropriate attraction to netrin-1 in the floor plate [38].

Interestingly, an interaction between DCC and Robo receptors was also found in the axons of the corpus callosum, but, unlike in the spinal cord, it functions here in the reverse direction, leading to inhibition of Slit/Robo signaling by netrin/DCC. Silencing of Slit/Robo repulsion enables precrossing callosal axons to approach and traverse the midline. After crossing, DCC is downregulated, activating Robo signaling, which expels axons from the midline [41].

Two recent studies have addressed the complicated crosstalk between different members of the semaphorin family. Sema3A and Sema3C are both expressed in the developing cortex with a certain degree of overlap (Figure 1d). *In vitro* assays demonstrated that Sema3C has attractive properties for cortical axons, whereas Sema3A is repulsive, raising the question as to how cortical axons react when they encounter both signals at the same time [42]. Application of mixtures of Sema3A and Sema3C to cortical explants suggested that Sema3A is the dominant cue, because it can suppress Sema3C-mediated attraction even when present at much lower concentrations than Sema3C. The proposed mechanism for integration of these opposing cues involves different compositions of neuropilin receptor subunits used by the two ligands. Sema3A only binds to neuropilin-1 homodimers, which transmit a strong repulsive response through their associated plexin co-receptors, whereas Sema3C interacts preferentially with neuropilin-1/neuropilin-2 heterodimers, eliciting a weaker attractive response via different plexins [43]. Similarly, several class 3 semaphorins are found in the developing limb, where their expression outlines the motor nerve trajectories (Figure 1a) [44–46]. It is therefore believed that Sema3 proteins are among the cues that channel extending motor axons into their pathways by surround repulsion. Like in cortical neurons, the magnitude of the response to different Sema3s is determined by the set of neuropilin receptors present on the growth cones. A recent study demonstrated that the balance between neuropilin-1 and neuropilin-2 depends on the levels of Sema3C expressed by motor neurons [46]. The presence of Sema3C affects the two receptors in opposite ways, decreasing the amount of neuropilin-1 and upregulating neuropilin-2. This lowers the responsiveness of axons to exogenous Sema3A and Sema3C and sensitizes them to Sema3F (Figure 3c). Sema3C gain and loss of function resulted in a change in surface neuropilin levels, suggesting that Sema3C might act by modulating receptor trafficking [46].

### *Synergistic interactions*

Limb-innervating motor axons of the $LMC_L$ population are attracted to the dorsal limb mesenchyme by GDNF and EphAs (Figure 1a) [5,6,9,10]. In fact, both GDNF and EphAs act synergistically through a common signaling receptor, the GDNF receptor Ret. EphrinAs, which in this case serve as receptors for EphAs, lack intracellular sequences and therefore need a transmembrane co-receptor to transmit signals into the cell. It was shown that

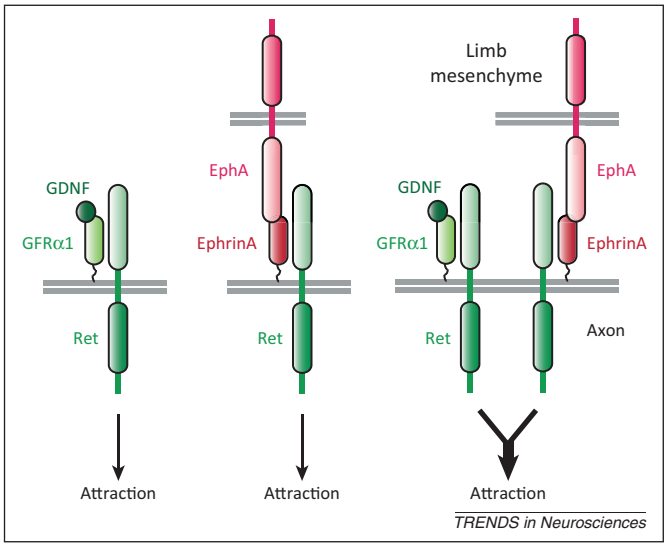


**Figure 4.** Synergy between glial-cell-derived neurotrophic factor (GDNF) and EphAs in motor axon guidance. GDNF (left) and EphAs (middle) induce chemoattraction in $LMC_L$ motor neurons by binding to their respective glycosylphosphatidylinositol (GPI)-anchored receptors GFRα1 and ephrinAs, which both signal through the transmembrane co-receptor Ret. $LMC_L$, lateral cells of the lateral motor column. Right: simultaneous binding of GDNF and EphAs elicits a much stronger attractive response [9].

ephrinAs co-localize and directly interact with Ret. Growth-promoting effects of EphAs on motor axons were enhanced by addition of GDNF and were abolished in explants from Ret mutant embryos. Moreover, subthreshold concentrations of the two ligands triggered a robust turning response of LMC axons when added together. On the basis of these data, it was proposed that Ret functions as a coincidence detector that integrates two different attractive signals, ensuring a stronger response when axons are exposed to both EphAs and GDNF (Figure 4) [9].

### *Permissive interactions*

In the previous sections we addressed interactions between *bona fide* guidance cues. In addition, several examples have been described where permissive factors, which themselves might not provide directional information, are required to enable axons to properly interpret instructive cues. The permissive factor either sensitizes axons to guidance molecules or switches their responses between attraction and repulsion.

As mentioned above, commissural axons in the spinal cord become sensitive to repellent cues after crossing the midline (Figure 1b) [12]. It was proposed that several molecules found at the midline switch on axon responsiveness to repellents of the Sema3 class. Precrossing commissural axons do not react to Sema3B because of the low levels of its signaling receptor component plexin-A1, resulting from plexin-A1 cleavage by the protease calpain-1. This protease is inactivated when axons reach the midline, allowing for accumulation of plexin-A1 on the membrane [47]. NrCAM and GDNF were identified as midline factors necessary for calpain-1 inhibition. It is known that NrCAM is cleaved, and soluble NrCAM ectodomain was sufficient to increase plexin-A1 levels on the growth cones and to change axon sensitivity to Sema3B, suggesting that NrCAM acts as a diffusible factor in this context [47]. GDNF acts via its alternative receptor neural cell adhesion molecule (NCAM), and not via Ret (Figure 5a). *In vitro* experiments with subthreshold amounts of NrCAM and GDNF suggested a synergistic relationship between these two permissive molecules [48], but further experiments will be needed to determine the nature of their cooperation. Another study showed that exposure to Shh sensitizes rat commissural axons to Sema3B and Sema3F [49]. This effect of Shh appears to be due to inhibition of cAMP/protein kinase A (PKA) signaling (Figure 5b), because application of adenylyl cyclase activator to spinal cord explants reduced Sema3 repulsion and caused axon path-finding errors similar to those seen in neuropilin-2 mutants and with a Shh function-blocking antibody [49]. It remains to be investigated whether Shh has overlapping functions and is involved in any crosstalk with NrCAM and GDNF, or whether the effects of these molecules are specific to certain subpopulations of commissural axons.

It was shown that interplay between three molecules, Sema6D, plexin-A1, and NrCAM, is required for guidance of crossed RGC axons at the optic chiasm [34,35] (Figure 1c). All three cues are present in the chiasm region, where Sema6D and NrCAM are co-expressed on midline glia, and plexin-A1 is expressed on chiasm neurons. *In*

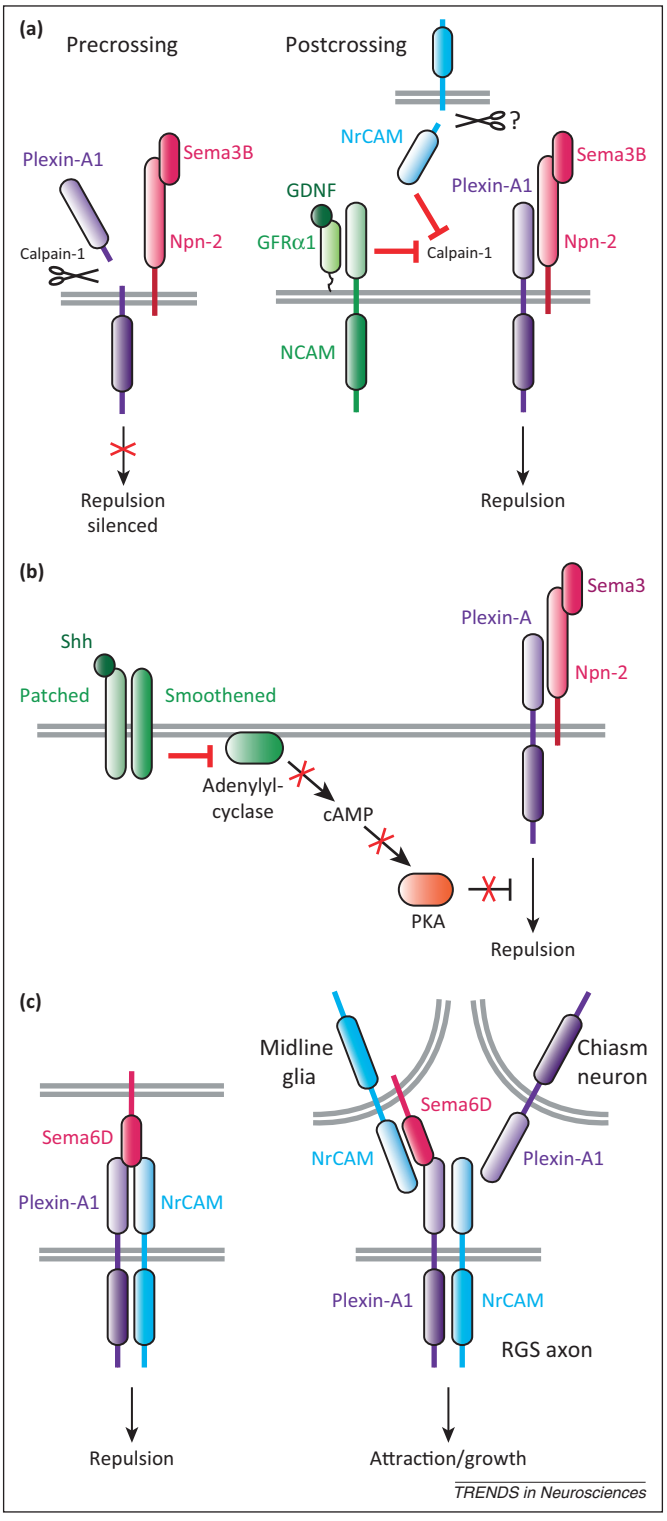


**Figure 5**. Permissive interactions between guidance cues. **(a)** Left: precrossing commissural axons are insensitive to Sema3B because of cleavage of the plexin-A1 receptor by the protease calpain-1. Right: in the floor plate, calpain-1 activity is inhibited by Ng-CAM-related cell adhesion molecule (NrCAM) and glial-cell-derived neurotrophic factor (GDNF), leading to an increase in plexin-A1 levels and sensitization of the axons to Sema3B repulsion [47,48]. NrCAM is presumably cleaved and acts as a soluble molecule [47]. The activity of GDNF is mediated by its receptor NCAM [48]. **(b)** Sonic hedgehog (Shh) was also implicated in switching on the sensitivity of commissural axons to class 3 Semas. Shh acts through Patched and Smoothened to lower cAMP levels and reduce protein kinase A (PKA) activity, which allows Sema3 repulsion [49]. **(c)** Left: contralateral retinal ganglion cell axons respond to Sema6D with repulsion. Plexin-A1 and NrCAM serve as Sema6D receptors on the axons. Right: the presence of NrCAM and plexin-A1 in the target tissue changes the activity of Sema6D into a growth-promoting effect. Plexin-A1 is expressed in a different population of cells and appears to act *in trans* with respect to Sema6D [35].

*vitro* assays demonstrated that Sema6D alone has a repulsive effect on crossed RGC axons. However, the presence of NrCAM and plexin-A1 converts this inhibitory action of Sema6D into a growth-promoting activity. Plexin-A1 and NrCAM detected on crossed RGC axons in turn serve as receptors for this tripartite ligand complex. Although the exact molecular mechanism of cooperation between the three cues remains to be clarified, plexin-A1 at the chiasm seems to function *in trans* in relation to the Sema6D/NrCAM complex, because it is expressed in a different population of cells, and soluble plexin-A1 extracellular domain was sufficient for its permissive effects (Figure 5c) [35].

An interesting case of combined netrin-1/Slit1 effects was observed in the thalamocortical projection, where Slit1 plays a permissive role in topographic mapping of a subset of thalamocortical axons [50]. Both netrin-1 and Slit1 are detected in a rostral high–caudal low gradient in the so-called corridor, where initial sorting of thalamocortical fibers occurs (Figure 1e). In thalamic explant culture experiments, Slit1 repelled rostral thalamocortical axons when applied alone, whereas netrin-1 alone had no effect. Surprisingly, a combination of the two molecules evoked an attractive response. Further experiments indicated that in this situation Slit1 does not provide directional information for axons, but allows their attraction to netrin-1 [50]. How can the same combination of guidance cues result in such different outcomes as observed for Slit and netrin-1 in commissural, callosal, and thalamocortical axons [37,38,41,50]? This might be because of multiple reasons, such as different relative concentrations of guidance factors or different combinations of receptors or their downstream signaling effectors expressed by the various neuronal populations. For several guidance molecules, opposite axonal responses have been observed, depending on the concentration of the cue. For instance, ephrinA2 and Shh both promote RGC axon outgrowth at low concentrations, but become inhibitory at high concentrations [51,52]. The intracellular mechanisms underlying these bimodal effects are not yet entirely understood, but a recent study has implicated local protein synthesis in responses to particular concentrations of cues [53]. The variability of responses depending on cue concentration might add further complexity to the integration of multiple guidance signals by the axons.

## Concluding remarks

The use of multiple signaling systems diversifies the possible guidance responses to a limited number of guidance cues, increases the fidelity, and reduces noise in guidance decisions. Although many examples of cue interactions have now been reported, we are far from understanding all the intricacies of the crosstalk between different cues and receptors. One difficulty in exploring the roles of combinations of molecules *in vivo* is that it requires the use of complex genetics to generate compound mutants. In addition, quantitative *in vitro* assays have not yet been fully exploited and should be used more extensively to gain insights into the interactions suggested by the genetic data.

Further studies will be necessary to decipher the molecular mechanisms of interactions between guidance systems, which are just beginning to be unraveled. Moreover, it remains to be investigated whether the integration of guidance signals only takes place locally in the growth cones or whether some of the signals might be transmitted retrogradely to the neuronal cell body. The cellular sources of the cues and their exact spatial distribution *in vivo* are still largely unexplored.

Significant advances in the area of guidance cue integration will be possible with the use of new methods. On the one hand, we need more information about the behavior of growing axons in their native surrounding. Live imaging of navigating axons *in vivo* [54] or in largely intact tissue preparations [55] will be useful for observing growth cone behaviors at pathway decision points in real time, and how they change as a result of genetic manipulations. In parallel, more sophisticated *in vitro* techniques are being developed for studying axonal pathfinding, including various microfluidic devices. Compared to classical cell culture methods, microfluidic chambers and other engineered culture substrates allow more faithful approximation of the complex microenvironments encountered by axons *in vivo*, providing very precise spatial and temporal control over the cues presented to the cells [56–59]. Combined with high-resolution microscopy, this can provide valuable new insights into the intracellular processes underlying axonal behaviors. Moreover, compartmentalized chambers can be used to isolate axons for imaging, biochemical, and expression analyses [53,60–62].

As more examples of guidance cue interactions are found and their mechanisms are described in greater detail, we might be able to uncover some common logic behind these interactions. Defining these general principles will be crucial for our understanding of neural circuit assembly *in vivo* and might help in the design of neural regeneration strategies.

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